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Hybridization between *Calopteryx splendens* and *C. haemorrhoidalis* confirmed by morphological and genetic analyses

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Hybridization between *Calopteryx haemorrhoidalis* and any of its congeners has not been reported until now. We observed spontaneous matings between male *C. splendens* and female *C. haemorrhoidalis* at a locality in Central Italy, together with some putative hybrid individuals that had a mixed phenotype. Here, we report the morphological and molecular characterization of five suspected hybrids collected from this population during 2001 ($n = 1$), 2012 ($n = 2$) and 2013 ($n = 2$). A discriminant analysis based on 13 morphological variables correctly separated both parental species (with 100% assignation success) and classified the hybrid from 2001 as *splendens* phenotype and those from 2012 and 2013 as *haemorrhoidalis*. Genotype data (microsatellite loci) was used to confirm the hybrid origin of these specimens, although there were differences between the individual from 2001 and those from 2012 and 2013; the 2001 individual had alleles that were present in both parent species, suggesting it is an F1 hybrid, but the individuals collected in 2012 and 2013 had private alleles at eight (out of 12) loci and only a small portion of the genome in common with *C. splendens*, which suggests that introgression is occurring in this population. Similarities in mitochondrial DNA sequences indicate that the 2001 hybrid and the 2012–2013 hybrids have *splendens* and *haemorrhoidalis* maternal origins respectively, which, in contrast with behavioural observations, indicates that interspecific matings in both directions are possible. This is the first demonstration that *C. haemorrhoidalis* can hybridize with other congeners to produce viable offspring.

Keywords: Odonata; dragonfly; Calopterygidae; sympatric populations; discriminant analysis; microsatellites; hybrids; introgression

Introduction

Hybridization, defined as reproduction between genetically distinguishable groups or taxa that produces viable offspring, occurs in at least 10% of animal species (Mallet, 2005). Although this phenomenon has been well documented in natural populations, hybridization has often been considered as generally unimportant for the biology of many species. More recently, however, the extent and genetic importance of hybridization is becoming apparent; for example as it can lead to introgression and sometimes to hybrid speciation (Mallet, 2008).

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Most observations on hybridization have been obtained from birds and plants, and less is known about the occurrence of this phenomenon among insect species, with the exception of well-studied groups such as lepidopterans and some *Drosophila* species (reviewed by Mallet, 2005). In odonates, precopulatory tandems and matings between heterospecifics have frequently been reported (e.g. Asahina, 1974; Bick & Bick, 1981; Corbet, 1999; De Marchi, 1990; Dumont, Heidari, & Atamuradov, 1997; Hayashi, Dobata, & Futahashi, 2004; Jordan, Simon, & Polhemus, 2003; Lindeboom, 1996; Monetti, Sánchez-Guillén, & Cordero Rivera, 2002; Utzeri & Belfiore, 1990; Weichsel, 1985), but confirmation that this behaviour yields successful interspecific hybrid offspring, as verified by genetic methods or breeding experiments, is confined to a handful of studies (e.g. Hayashi, Dobata, & Futahasi, 2005; Keränen, Kahilainen, Knott, Kotiaho, & Kuitunen, 2013; Lindeboom, 1996; Monetti et al., 2002; Sánchez-Guillén, Van Gossum, & Cordero Rivera, 2005; Tynkkynen et al., 2008).

The taxonomic status and relationships of European members of the genus *Calopteryx* are quite well studied, with contemporary species thought to have originated from two main clades: (1) the *virgo*/*haemorrhoidalis* group that is about 5.3 My old and with *C. haemorrhoidalis* thought to be the youngest species (derived some 2.4 My ago); and (2) the *splendens* group that is about 3.7 My old (Dumont, Vanfleteren, De Jonckheere, & Weekers, 2005; Misof, Anderson, & Hadrys, 2000; Weekers, De Jonckheere, & Dumont, 2001). Reproductive isolation within *Calopteryx* is not complete and hybridization is an apparently common phenomenon between many species and subspecies of this genus where they occur in sympatry, such as *C. xanthostoma* and *C. splendens* in southern France (Dumont, Mertens, & De Coster, 1993), *C. xanthostoma* and *C. splendens caprai* in the north of Italy (Weekers et al., 2001) and *C. splendens* and *C. virgo* in southern Germany (Lindeboom, 1996) and Finland (Keränen et al., 2013; Tynkkynen et al., 2008). Besides an anecdotal description of a possible hybrid between *C. haemorrhoidalis* and *C. virgo meridionalis* from a locality in the North of Italy (Terzani, 1993), *C. haemorrhoidalis* is the only European species within the genus *Calopteryx* that is not known to hybridize with any of its congeners (Weekers et al., 2001).

Moreover, identification of hybrid individuals within *Calopteryx* is mostly based on morphological descriptions (e.g. Dumont et al., 1997; Terzani, 1993), and only two studies have confirmed the status of suspected hybrid individuals using genetic markers (Keränen et al., 2013; Tynkkynen et al., 2008). Here, we use a combination of microsatellite genotyping, mitochondrial DNA sequencing and a discriminant analysis of phenotype to identify the specific origin of five suspected *C. splendens*–*C. haemorrhoidalis* hybrids that were collected in different years at a location in Central Italy. We provide the first unambiguous evidence that *C. haemorrhoidalis* can hybridize with other species of its genus.

Materials and methods

Study species, study site and sample collection

Calopteryx splendens and *C. haemorrhoidalis* are riverine odonates, whose ranges overlap in mid-Europe (Dijkstra & Lewington, 2006). Both species are distinguished mainly by the conspicuous coloration of males. Males of *C. splendens* are metallic blue with a blue band in each wing, and metallic blue veins; the underside of S8–10 in this species is bright yellow to greyish white. *Calopteryx haemorrhoidalis* males are characterized by their reddish-bronze or black body coloration and largely dark wings, and the underside of the abdomen tip appears uniformly bright pink to vivid red (Dijkstra & Lewington, 2006). Furthermore, the venation density is much higher in *C. splendens* than in *C. haemorrhoidalis* (see Figure 1E, F). *Calopteryx splendens* females have metallic green body and clear greenish wings with green venation, a character

Table 1. Summary of the morphological variables measured on *Calopteryx splendens*, *C. haemorrhoidalis*, and the five putative hybrids.

Variable	*C. splendens* ($n = 17$)	*C. haemorrhoidalis* ($n = 16$)	Putative hybrids ($n = 5$)
Forewing length (FL)	27.52 ± 0.27	28.06 ± 0.25	28.00 ± 0.31
Hind wing length (HL)	26.59 ± 0.22	27.17 ± 0.25	26.91 ± 1.55
Forewing width (FW)	8.05 ± 0.10	8.32 ± 0.08	8.78 ± 0.19
Forewing spot (FS)	20.01 ± 0.32	25.47 ± 0.24	23.39 ± 1.55
Hind wing spot (HS)	18.93 ± 0.23	24.16 ± 0.28	22.25 ± 1.41
FS/FL*	0.73 ± 0.01	0.91 ± 0.004	0.84 ± 0.06
HS/HL*	0.71 ± 0.01	0.89 ± 0.005	0.83 ± 0.05
Forewing cells anal loop (FC)	3.82 ± 0.20	2.44 ± 0.13	3.20 ± 0.20
Hind wing cells anal loop (HC)	4.18 ± 0.18	3.00 ± 0.13	4.00 ± 0.32
Average cells anal loop (CAL)**	4.00 ± 0.15	2.72 ± 0.05	3.60 ± 0.19
Number veins Cu-A (forewing) (V)	13.18 ± 0.30	11.31 ± 0.27	12.20 ± 0.49
Paraproct length (PL)	1.61 ± 0.02	1.63 ± 0.02	1.65 ± 0.02
Paraproct width (PW)	0.75 ± 0.01	0.69 ± 0.01	0.71 ± 0.004
Cercus length (CL)	0.69 ± 0.01	0.62 ± 0.01	0.68 ± 0.02
Cercus width (CW)	0.29 ± 0.02	0.27 ± 0.004	0.29 ± 0.01
Ninth segment length (9L)	1.49 ± 0.04	1.60 ± 0.02	1.56 ± 0.04
Genital pore flaps (G)	0.55 ± 0.01	0.58 ± 0.01	0.59 ± 0.03

Note: Listed are the mean values (±SE) for each variable. All lengths are given in mm. The variables that were included in the discriminant analysis are presented in bold. For details on how these variables were measured, see Figure 1.
*Wing spot size was estimated as the ratio between spot length (FS and HS) and wing length (FL and HL).
**For the number of cells in the anal loop (CAL), only the average value between both wings was used in the discriminant function.

that allows easy separation from other congeneric species. Females of *C. haemorrhoidalis* are metallic green to bronze and can be distinguished by their contrasting dark hind wing tips (a character that shows high intrapopulation variability) and narrow pale humeral lines (Dijkstra & Lewington, 2006).

Fieldwork was done at the river Forma Quesa, near Pontecorvo, in Central Italy (41°25'43.8″ N, 13°39'36.7″ E), where *C. haemorrhoidalis* is the most common odonate species, but it nonetheless coexists with a large population of *C. splendens* and a smaller number of *C. virgo*. This river has cold water (15–18 °C), probably due to the presence of subterranean sources, and has a good riparian forest in the last kilometre (with species from the genera *Alnus*, *Populus*, *Acer* and *Salix*), before it ends at the river Liri.

Spontaneous matings between male *C. splendens* and female *C. haemorrhoidalis* have been observed in this population (ACR, pers. obs.). Five suspected hybrid individuals, showing a mixed phenotype between these two species, were collected during different years: one in 2001, two in 2012, and the last two individuals in 2013.

Morphological discriminant analysis

We measured 17 morphological variables from wings and anal appendages, in a sample of 16 males of *C. haemorrhoidalis* and 17 males of *C. splendens*, as well as in the five putative hybrids (see Table 1, Figure 1). To maximize the phenotypic variability of the dataset, measurements were taken in a sample of each of the parental species collected at the river Forma Quesa over a range of years (2001, 2004, 2007, 2010 and 2012).

The software ImageJ (Abramoff, Magalhaes, & Ram, 2004; available at http://imagej.nih.gov/ij/) was used to take measurements on images of wings (scanned at 600 dpi using a Canoscan 9000F scanner), and on the abdomens (photographs taken with a Leica MC170 digital camera attached to an Olympus SZ60 stereomicroscope).

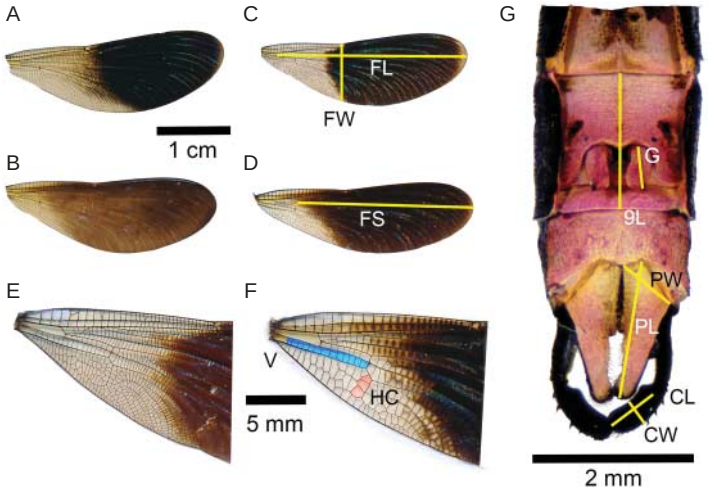

Figure 1. Morphological variables used in the discriminant analysis, and how they were measured. Right forewing of (A) hybrid H1 and (B) hybrid H3; (C) a wing scan of a *Calopteryx splendens* male, showing how wing length (FL) and width (FW) were measured; (D) wing scan of a *C. haemorrhoidalis* male, showing how the spot length (FS) was measured. Detail of (E) the hind wing of a *C. splendens* male and (F) a *C. haemorrhoidalis* male; with the number of cells in the anal loop (HC) highlighted in red. The number of cross veins between the cubitus and anal vein on the forewing was counted on the forewing as highlighted in blue. (G) Ventral view of the last abdominal segments of hybrid H1, showing how the length of ninth abdominal segment (9L), the length of the genital pore flaps (G), the paraproct length (PL) and width (PW), and cercus length (CL) and width (CW) were measured.

Thirteen of these morphological variables were used to calculate a discriminant function using xlStat 2013.1 (www.xlstat.com). Data of measurements made on males from both parental species was first used to estimate the discriminant function, and then the hybrids were entered and classified using the same function.

DNA extraction, microsatellite genotyping and mtDNA amplification

To provide a representative "genetic signature" of each parental species, 24 individuals of *C. splendens* and *C. haemorrhoidalis* were collected during 2007 and 2010 in Pontecorvo. All animals were collected with a hand net and stored in absolute ethanol at 4 °C prior to DNA extraction. Whole genomic DNA was isolated from thoracic muscle using either a standard CTAB protocol (Doyle & Doyle, 1987) or the DNeasy Tissue kit (Qiagen, Inc., Venlo, Netherlands), following the manufacturer's instructions.

We first tested whether the 19 microsatellite loci developed for *C. splendens* and *C. virgo* (Molecular Ecology Resources Primer Development Consortium, 2011) would PCR-amplify samples of DNA from *C. haemorrhoidalis*. The 12 loci that gave positive amplification in *C. haemorrhoidalis* were then used to genotype all samples. PCRs were carried out in a GenAmp PCR system 9700 (Applied Biosystems, Foster City, California, USA) in 10 µl reaction volumes, each containing 1xGoTaq®Green Master Mix (Promega, Fitchburg, Wisconsin, USA), 1× BSA (10 mg ml^{-1}), 3 mM $MgCl_2$, 0.2 pmol of each primer and ~10–50 ng of DNA. PCR profiles were: 3 min at 94 °C, followed by 35 cycles of [92 °C 30 s, 58 °C 30 s, 72 °C 30 s], and 5 min at 72 °C. PCR products were pooled in one of three genotyping pools, determined by allelic size range and the 5′ fluorescent dye, along with a GeneScan500 LIZ Size Standard (Applied Biosystems), and separated by capillary electrophoresis on an ABI3130xl Genetic Analyzer (Applied Biosystems). Alleles were sized using GeneMapper v.3.7 software (Applied Biosystems).

To identify the maternal species origin of the suspected hybrids, we amplified a 610 bp fragment of mitochondrial DNA (mtDNA), spanning part of the 16S, $tRNA^{Leu}$ and part of the NADH region 1, using primers P850 [5′-TTC AAA CCG GTG TAA GCC AGG-3′], and P851 [5′-TAG AAT TAG AAG ATC AAC CAG C-3′] (Abraham et al., 2001) in the five suspected hybrids, as well as in four pure *C. splendens* and four pure *C. haemorrhoidalis*. PCRs were carried out in a GenAmp PCR system 9700 (Applied Biosystems) in 10 µl reaction volumes containing 1× GoTaq®Green Master Mix (Promega), 1× BSA (10 mg ml^{-1}), 3 mM $MgCl_2$, 0.2 pmol of each primer and ~10–50 ng of DNA. PCR profiles were: 4 min at 95 °C, 35 cycles of [95 °C 45 s, 53 °C 45 s, 72 °C 1 min], and 5 min at 72 °C. PCR products were purified using 0.6 units of *Exonuclease* I and 0.75 units of *Antarctic Phosphatase* (New England Biolabs, Ipswich, Massachusetts, USA), sequenced in both directions using BigDye v.3.1 chemistry (Applied Biosystems) and capillary electrophoresis on an ABI3130xl (Applied Biosystems).

Microsatellite data analyses

We used Arlequin v.3.01 (Excoffier, Laval, & Schneider, 2005) to test for departure of Hardy–Weinberg equilibrium (HWE), and to estimate genetic differentiation (F_{st}) between *C. splendens* and *C. haemorrhoidalis*; the significance of F_{st} was assessed by 10,000 permutations. Basic measures of genetic diversity, namely the number of alleles (N_a) and private alleles (P_a), were calculated using GenAlex v.6.1 (Peakall & Smouse, 2006).

We used Hybridlab v.1.1 (Nielsen, Bach, & Kotlicki, 2006) to create samples of artificial hybrids. This software creates multilocus F1 hybrid genotypes between two samples by randomly drawing alleles as a function of their frequency distributions and linkage equilibrium and assuming random mating; we simulated datasets for 24 genotypes of each of the following crosses: F1 individuals (*C. splendens* × *C. haemorrhoidalis*), first backcross with *C. splendens* (1 CsB, F1 × *C. splendens*), first backcross with *C. haemorrhoidalis* (1 ChB, F1 × *C. haemorrhoidalis*), second backcross with *C. haemorrhoidalis* (2 ChB, 1 ChB × *C. haemorrhoidalis*), third backcross with *C. haemorrhoidalis* (3 ChB; 2 ChB × *C. haemorrhoidalis*), fourth backcross with *C. haemorrhoidalis* (4 ChB; 3 ChB × *C. haemorrhoidalis*), and fifth backcross with *C. haemorrhoidalis* (5 ChB; 4 ChB × *C. haemorrhoidalis*).

The Bayesian model-based clustering of multilocus genotype data implemented in Structure v.2.3.4 (Pritchard, Stephens, & Donelly, 2000) was used to assign hybrid individuals to either potential parent species. We used the "prior population information model" to assign each of the suspected hybrids, as well as the simulated hybrid genotypes to each of the two clusters corresponding to the reference groups (i.e. *C. haemorrhoidalis* [$n = 24$] or *C. splendens* [$n = 24$]); next, we evaluated the admixture proportions (±90% confidence intervals) of each hybrid individual (i.e. the 168 artificial hybrids and the five putative hybrids). We conducted 10 independent runs of Structure for $K = 2$ (i.e. two parental species), with each run comprising 100,000 Markov chain Monte Carlo (MCMC) iterations, after a burn-in period of 20,000 MCMC iterations, using the admixture model and correlated allele frequencies.

Sequence data analysis

Forward and reverse mtDNA sequences for each individual were assembled and manually edited in Geneious v.7.1.4 (www.geneious.com), and consensus sequences were aligned using ClustalW (Thompson et al., 1994) as implemented in Geneious v.7.1.4. All variable positions were confirmed by visual inspection and only high quality traces were considered. All sequences were deposited in Genbank (www.ncbi.nlm.nih.gov/genbank) under accession numbers KM224434–46. Phylogenetic relationships among mtDNA sequences were reconstructed

Figure 2. Spontaneous interspecific mating between a male of *Calopteryx splendens* and a female of *C. haemorrhoidalis* observed at the study locality in Pontecorvo on August 2008.

using the neighbour-joining (NJ) method (Saitou & Nei, 1987) implemented in MEGA v.6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), using *Vestalis melania* as outgroup (Genbank Acc. No. JX050224; Chen et al., 2014).

Results

Spontaneous matings between males of *C. splendens* and females of *C. haemorrhoidalis* in Pontecorvo were rare, comprising only 0.5% of the 202 matings observed in 1999, and 1.6% of 123 matings observed in 2000, two years in which intensive behavioural observations were conducted for other purposes (Cordero Rivera & Andrés, 2002). Interspecific matings were also occasionally observed in other years (Figure 2), although no quantitative data are available.

Morphological description of the hybrids and discriminant analysis

All five putative hybrid individuals found were male, and presented a mixture of the phenotypic characteristics of *C. haemorrhoidalis* and *C. splendens*: the hybrid captured in 2001 was a mature male, with wing coloration similar to *splendens*, and a dense wing venation, also characteristic of *splendens*. Body colour was a mixture between both species, with green and reddish metallic reflections when alive. The ventral surface of the last abdominal segments showed a reddish-carmine coloration, similar (although not so intense) to *haemorrhoidalis* (Figure 3A).

The two hybrids captured in 2012 were almost mature, and showed also the reddish ventral coloration typical of *haemorrhoidalis* in the last abdominal segments, although not yet fully developed. The two hybrids from 2013 were young individuals, and showed a yellowish ventral coloration in the last abdominal segments, typical of *splendens*, but also with some red colour as *haemorrhoidalis*. All individuals from 2012 and 2013 showed wing pigmentation and venation density more similar to *splendens* than to *haemorrhoidalis*, and body coloration was black-bronze with greenish (abdomen) and reddish (thorax) reflections (Figure 3B).

Table 1 summarizes the morphological variables measured. The two species are very similar, particularly in the size of genital pore flaps (which are the rudiments of primary genitalia) and anal appendages. The 15 measured variables were reduced to 13 for the discriminant function, because wing spot size was entered as a proportion, and the number of cells in the anal loop was averaged over forewings and hind wings. The calculated discriminant function correctly

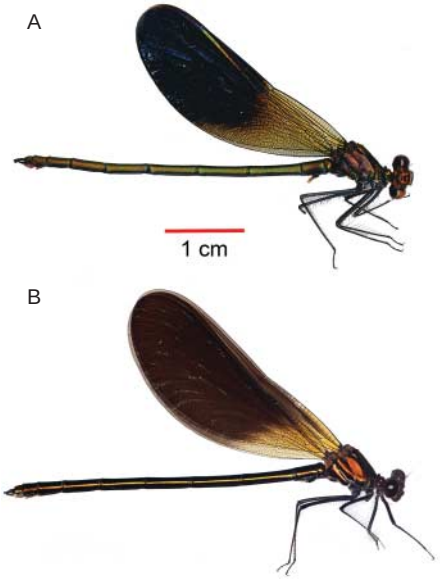

Figure 3. Phenotype of (A) the hybrid male H1, collected in 2001; and (B) the hybrid male H4, collected in 2013.

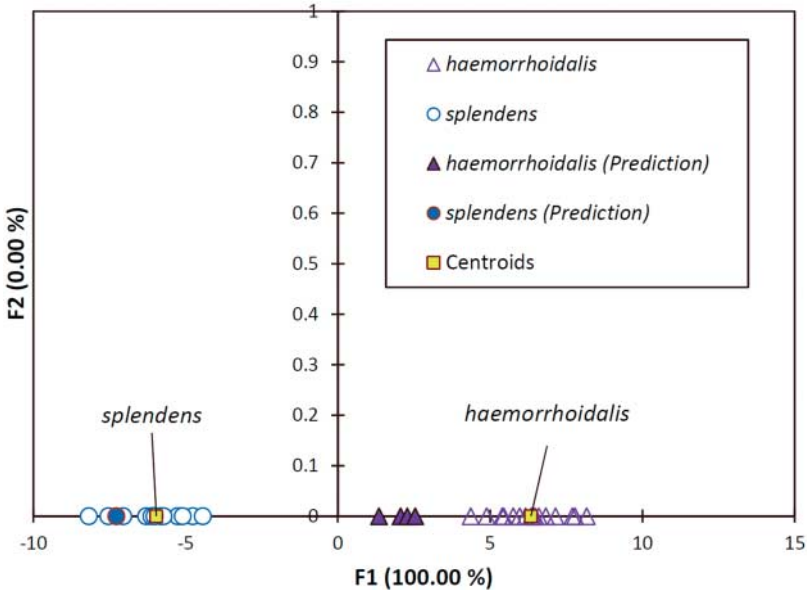

Figure 4. Results of a discriminant analysis based on 13 morphological variables measured on *Calopteryx splendens*, *C. haemorrhoidalis*, and the five hybrids collected at Pontecorvo. Using a single function (F1) both species are correctly separated. The hybrids are assigned to *splendens* (hybrid H1, closed dot on the left of the plot) or to *haemorrhoidalis* (H2–H5, closed triangles on the right).

separates both parental species, with no errors in classification. The hybrid from 2001 was classified as *C. splendens*, whereas the remaining four hybrids were classified as *C. haemorrhoidalis* (Figure 4). In relation to parental species, hybrids were intermediate in most morphological characters measured, but the average width of the wings was higher in hybrids (see Table 1).

Table 2. Summary of microsatellite allele diversity in *Calopteryx splendens*, *C. haemorrhoidalis*, and the five putative hybrids collected at Pontecorvo.

	C. splendens ($n = 24$)				*C. haemorrhoidalis* ($n = 24$)				Hybrid 2001 ($n = 1$)			Hybrids 2012–2013 ($n = 4$)		
Locus	N_A	P_A	Size	H_E	N_A	P_A	Size	H_E	N_A	P_A	Size	N_A	P_A	Size
Cv7	2	1	367–372	0.083	1	0	372	—	1	0	372	3	2	372–381
Cv48	2	1	207–209	0.414	1	0	197	—	2	0	197–209	2	1	197–199
Cv60	2	0	236–239	0.467	1	0	231	—	2	0	231–236	3	0	231–239
Cs5	1	0	131	—	2	1	129–131	0.295*	1	0	131	1	1	128
Cs7	2	1	173–175	0.463	2	2	174–176	0.494	1	0	173	4	3	173–191
Cs10	1	0	190	—	1	0	188	—	2	0	188–190	1	0	188
Cs52	3	0	235–241	0.537*	3	0	235–241	0.442	2	0	237–241	5	1	235–245
Cs54	1	0	226	—	4	0	232–239	0.691	2	0	226–239	4	1	229–236
Cs66	1	0	231	—	1	0	220	—	2	0	220–231	1	0	220
Cs104	5	2	181–189	0.780	4	1	179–189	0.697*	1	0	185	5	2	176–189
Cs179	2	1	223–229	0.502*	2	0	225–226	0.510	2	0	223–225	3	1	221–226
Cs181	4	2	214–231	0.570	1	0	229	—	2	0	227–229	1	0	229

Note: Listed are: number of alleles (NA), number of private alleles (PA), allele size range (in bp), and expected heterozygosity (HE) for each of the 12 loci analyzed.
*indicates significant deficit of heterozygotes ($p < 0.05$).

Microsatellite data analyses

The number of alleles (N_A) per locus in *C. splendens* ranged from 1 to 5, and expected heterozygosity (H_e) ranged from 0.083 to 0.780, with private alleles (P_A, alleles unique to that species) present at 11 of the 12 loci. For *C. haemorrhoidalis*, N_A ranged from 1 to 4, H_e ranged from 0.295 to 0.697, and P_A were found at 10 loci (Table 2). Significant genetic differentiation between *C. splendens* and *C. haemorrhoidalis* ($F_{st} = 0.7$, $p < 0.001$) implies that the microsatellite loci have sufficient statistical power to detect hybrids.

Genetic differences were apparent between the putative hybrid individual collected in 2001 and those collected in 2012 and 2013: whereas the male from 2001 had alleles that were present in both parental species, suggesting that it could be a F1 hybrid, the individuals from 2012 and 2013 had alleles not found in either of the parental species at eight loci (Table 2). Given these results, and to discard any potential backcrossing with *C. virgo*, a species also present in this locality, we used the 12 microsatellite markers to genotype a sample of 24 individuals of *C. virgo* from the sample locality; however, PCR-amplification was successful only at four of the loci (*Cs54*, *Cv48*, *Cv60* and *Cs104*) and none of the alleles present at these loci were found in any of the putative hybrid individuals (data not shown).

When examining the admixture proportions of the artificial hybrids and backcrosses (see Supplementary Information), the F1 artificial hybrids showed admixture proportions of 55–41% to *C. haemorrhoidalis* and 59–45% to *C. splendens*; the 1CsB (first *C. splendens* backcross) showed 48–12% admixture proportions to *C. haemorrhoidalis* and 93–52% to *C. splendens*; and the five *C. haemorrhoidalis* backcrosses (1–5ChB) showed admixture proportions between 98 and 50% to *C. haemorrhoidalis*; and between 43 and 2% to *C. splendens*. The hybrid collected in 2001 (H1) was intermediate between the two main clusters (i.e. parental species), with 43% and 57% admixture proportions assigned to *haemorrhoidalis* and *splendens* respectively, suggesting it to be an F1 hybrid (Figure 5). The hybrids collected in 2012 and 2013 showed a skew in their admixture proportions, which varied between 85–97% of admixture to *haemorrhoidalis* and 4–15% to *splendens*, which implies a significant degree of introgression for these individuals (Figure 5).

C. splendens and *C. haemorrhoidalis* differ 14.2% at the partial ND1 sequence and exhibit no intraspecific variation in our samples, except for one single nucleotide polymorphism in one sample of *C. splendens*. The mtDNA of the hybrid collected in 2001 is of *C. splendens*

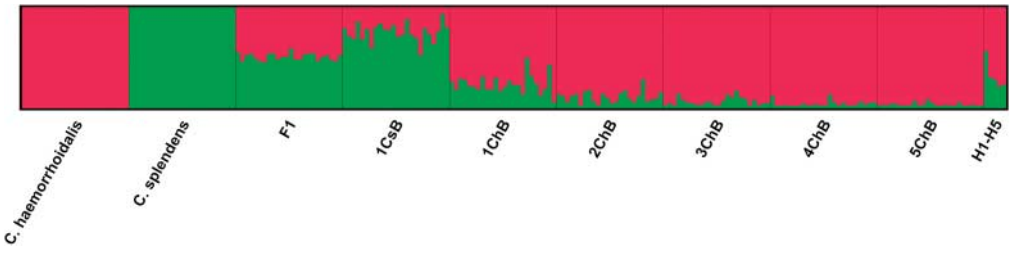

Figure 5. Estimated population structure from Bayesian structure analyses using the program STRUCTURE. Probabilities of assignment to each predefined cluster (pure *Calopteryx haemorrhoidalis* and pure *C. splendens*) for 168 simulated hybrid genotypes (F1, 1CsB, 1ChB, 2ChB, 3ChB, 4ChB, 5ChB; see main text for details), and the five putative hybrids (H1–H5) collected in Pontecorvo. Each bar represents a single individual, and the proportion of the bar that is red or green represents the proportion of assignment to cluster one (*C. haemorrhoidalis*) or cluster two (*C. splendens*), respectively.

maternal origin, whereas the mtDNA of the hybrids collected in 2012 and 2013 belongs to the *C. haemorrhoidalis* group (Figure 6).

Discussion

Hybridization plays an important role in evolution and speciation, and it has been frequently reported in several *Calopteryx* species, although it was not known to occur in *C. haemorrhoidalis*. Our integrated morphological and genetic analyses provide the first and unequivocal demonstration that hybridization between *C. haemorrhoidalis* and *C. splendens* can occur in natural populations, leading to viable offspring which in turn might backcross with the parental species.

Calopteryx is a genus where precopulatory courtship is rather elaborate (Córdoba-Aguilar & Cordero-Rivera, 2005), which would make instances of hybridization somewhat difficult in natural populations. However, once these behavioural barriers to tandem formation are surpassed, copulation can be easily achieved, given that this odonate family has simple anal appendages, which show little morphological differentiation among species (Adams & Herman, 1991; Corbet, 1999). In our case, we have also found that anal appendages are similar between *C. haemorrhoidalis* and *C. splendens* (Table 1), which presumably facilitates the success of the observed interspecific matings. Nevertheless, despite the observed similarities in genital morphology, our discriminant analysis indicates that the two species can be unambiguously separated using a combination of morphological variables (Figure 4), even if we do not take into account the different wing and body coloration of both species (blue and green in *splendens* and black in *haemorrhoidalis*). Hybridization produces phenotypes that are morphologically intermediate between the parental species, and hybrids show a different coloration, which makes them conspicuous and easy to detect in the field (see Figure 3).

Microsatellite data distinguish F1 hybrids from backcrosses. The admixture proportions of the hybrid collected in 2001 indicate that it is a clear F1 hybrid, whereas the admixture proportions of the four hybrids collected in 2012 and 2013 suggest that they are backcrossed individuals. Interestingly, these four individuals showed private alleles at eight of the microsatellite loci, which are very unlikely to be of *C. virgo* origin, since most loci failed to PCR-amplify in our sample of this species. The possibility exists that these alleles are present in the parental species, but that we have not found them in our samples because of limited sample size or because they were collected in a different year. Alternatively, these novel alleles may be an example of the so-called “rare allele phenomenon”, which refers to the situation when alleles that are rare or non-existent in the parental species arise at high frequencies in hybrid individuals (Sage & Selander, 1979). Despite being a quite widely described fact in animal hybrid zones

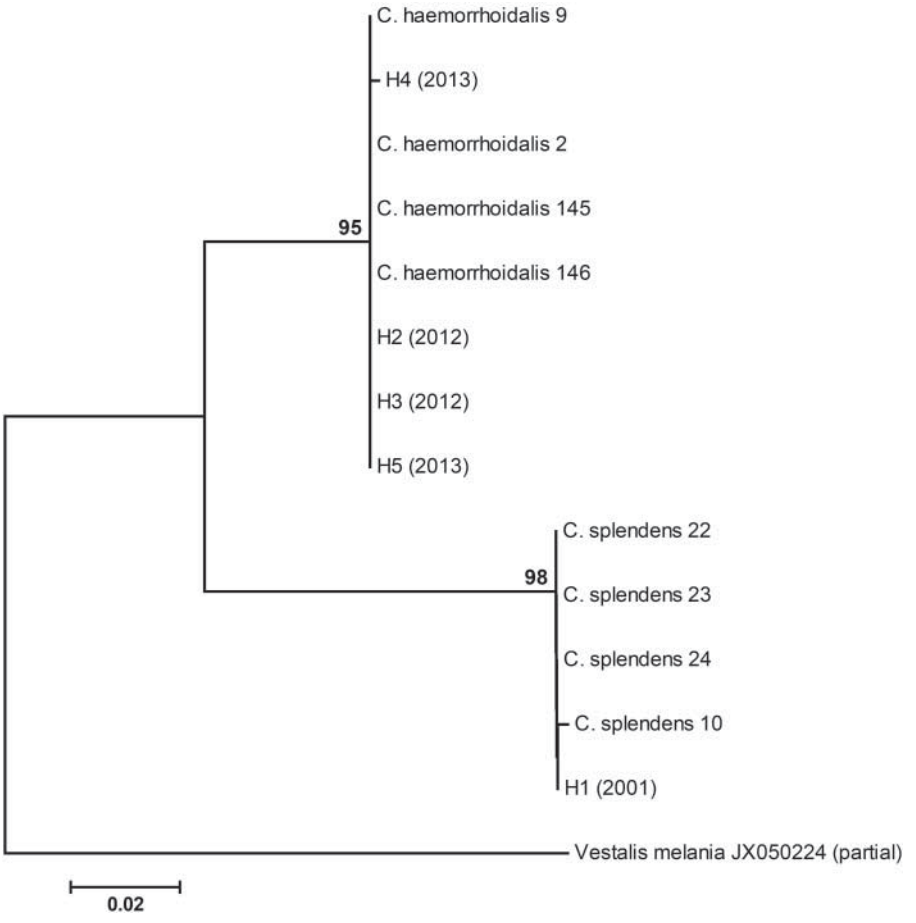

Figure 6. Neighbour-joining tree based on distances between mitochondrial NDI haplotypes, showing the relationships between each of the parental species and each of the five hybrid individuals collected at Pontecorvo. Values over branches indicate bootstrap support for each clade (1,000 replicates).

(e.g. Hoffman & Brown, 1995; Schilthuizen, Hoekstra, & Gittenberger, 1999; Steinmetz, Johannesen, & Seitz, 2004); it has never been reported for odonates. Genotyping a sample of *C. splendens* and *C. haemorrhoidalis* from 2012–2013 would help clarify the origin of the rare alleles present in the hybrids.

All hybrid individuals identified in this study were males. A reason why we did not detect any hybrid female could be that females of both species have a similar coloration, and thus any hybrid female that could be present in the population would not be readily distinguished without detailed inspection or a huge genetic sampling effort (e.g. see Keränen et al., 2013).

That the F1 hybrid from 2001 is of *splendens* maternal origin contrasts with our behavioural observations, in which all matings involved a male *C. splendens* and a female *C. haemorrhoidalis* (Figure 2). The implication of this finding is that interspecific matings in both directions are possible, which is in agreement with results of hand-pairing experiments performed between males and females of both species at the same study site, in which almost no female refuses copulation with a heterospecific male (Cordero-Rivera, unpublished data). Bidirectional hybridization has been also demonstrated for *C. virgo* and *C. splendens* in Finland (Keränen et al., 2013; Tynkkynen et al., 2008). On the other hand, all the identified backcrosses had *haemorrhoidalis* mtDNA, indicating that they are the product of a backcross with a *C. haemorrhoidalis* female.

The frequency of spontaneous hybrid matings that we observed is lower than the 2.3% and 6% mating frequencies reported for *C. splendens* and *C. virgo* in Finland (Tynkkynen et al. 2008 and Keränen et al. 2013, respectively). The most likely explanation for this is the difference in density between the two species in our study site (*C. haemorrhoidalis* is much more common than *C. splendens*), which implies fewer opportunities for interspecific encounters.

In conclusion, despite the apparent scarcity of interspecific matings in the study population, our results suggest that hybridization between *C. splendens* and *C. haemorrhoidalis* produces viable progeny, and the fact that we have found backcrossed individuals indicates that some F1 hybrids were sufficiently successful at mating with *C. haemorrhoidalis*. At low levels, introgression can be important as it can produce genetic variation that, in turn, can promote divergence and even speciation (Mallet, 2008). In fact, our backcrossed individuals showed private alleles at eight of the microsatellite loci, which suggests that copulation with heterospecifics in this population could bring some fitness benefits through the creation of new genetic variability and thus new adaptation.

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Supplemental data

Supplemental data for this article can be accessed via the online version [http://dx.doi.org/10.1080/13887890.2014.951696].

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